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The objective of the work is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapies brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer. Human cell-lines that over-express non-phosphorylatable mutants of CDC2 were used to establish the importance of inhibitory phosphorylation of CDC2 in checkpoint control in human cells. We have demonstrated that the activity of Cdc25, the phosphatase that activate Cdc2, is decreased in response to damaged DNA. We have identified a human homolgue of the checkpoint kinase, hChk1, that phosphorylates and inactivates Cdc25 *in vitro*. Experiments are underway to determine the exact molecular details by which DNA damage leads to inactivation of Cdc25.

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5)Introduction

The overall objective of the work proposed in this grant is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapy brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer.

6) Methodology and Results

Biochemical and cell biological techniques are being employed to address this problem. The three technical objectives that were defined in the proposed work are summarized below:

Technical Objective 1
Examine the pattern of expression and activity of mitotic checkpoint control proteins in transformed and non-transformed breast cell lines. (Equivalent to Task 1 in SOW 1-15 months)

Technical Objective 2
Examine the response of transformed and non-transformed breast cell lines to cytotoxic treatments in particular to correlate the operation of normal checkpoint controls with cell survival or death. (Equivalent to Task 2 in SOW 12-30 months)

Technical Objective 3
Determine the importance of checkpoint control proteins in cytotoxic sensitivity by genetic manipulation of WEE1, CDC25, Cyclin B and CDC2 *in vivo*.. (Equivalent to Task 3 in SOW 30-48 months)

As described in a previous annual report (94-95) our analysis of the pattern of expression and activity of mitotic checkpoint control proteins (CDC2, Cyclin A and B and WEE1) in transformed and non-transformed breast cell lines showed that there is over-expression of CDC2/Cyclin B in transformed breast cell-lines. However the level of over-expression was not very great and we concluded that it would not be feasible to analyse the viability of cells following cytotoxic treatment relative to these modest changes in expression level. We therefore decided to concentrate our efforts in pursuit of technical objective 3: That is, to determine the importance of checkpoint control proteins in cytotoxic sensitivity by genetic manipulation of WEE1, CDC25, Cyclin B and CDC2 *in vivo*. To do this we used the tTaHela cells system of Gossen and Bujard (1) that allows conditional expression of proteins under the control of a tetracycline repressible promoter. HeLa cells express the papillomavirus E6 protein which renders them defective for p53 function (2). Therefore HeLa cells provide a model cell-line in which analysis of G2 checkpoint is not complicated by the operation of the G1 checkpoint and the data we have gained from them is in itself an important contribution to the understanding of checkpoint control in the 50%-60% of breast cancers that lack p53.

A) ALTERATION IN CHECKPOINT CONTROL PROTEINS IN RESPONSE TO IRRADIATION.

In last years report we described the work we had done using ectopic expression of Cdc2 mutations which demonstrated the importance of inhibitory phosphorylation of Cdc2 in the

survival response of cells that have been exposed to agents which either damage DNA or that delay DNA replication. Since publication of these results we have continued with the experiment described in the original proposal that are aimed at characterizing alterations in the opposing kinases and phosphatases that regulate Cdc2 in response to DNA damage and/or replication blocks. We first developed an *in vitro* assay to look at changes in Cdc25 activity in response to irradiation

Dephosphorylation of Cdc2 is inhibited by DNA damage

As shown in Figure 1, the phosphorylation state and activity of Cdc2 was monitored in lysates prepared from HeLa cells. In the presence of EDTA, endogenous Cdc2 was found to activate spontaneously, over-time. Activation of Cdc2 correlated with a loss of the inhibited phosphorylated from of Cdc2, visualized as the slower migrating species on SDS-PAGE. Under these assay conditions, in which kinase reactions are inhibited by chelation of Mg^{2+} with EDTA, opposing kinase activity cannot affect the rate of dephosphorylation. As there is no gain of Cdc2 or Cyclin B protein (data not shown) and activation correlates with loss of the gel-mobility species that represent inhibited Cdc2, we reasoned that the increased activity of Cdc2 is the result of dephosphorylation. The same analysis was performed on lysates of HeLa in which the DNA had been damaged by exposure to 10 Grey of γ-irradiation from a cesium^{137} source 1 hour before harvesting. A significant reduction in the rate at which Cdc2 is dephosphorylated was reproducibly seen in extracts from irradiated cells. This experiment demonstrates that Cdc25 activity is depressed following irradiation.

In view of the genetic data that Chk1 kinases regulate Cdc2 phosphorylation in response to DNA damage (3, 4, 5) and considering the striking conservation of mitotic control mechanisms between fission yeast and humans we reasoned that if a Chk1 homologue exists in human cells it might be a component of the checkpoint that regulates dephosphorylation of Cdc2 in response to irradiation.

Human Chk1 is structurally related to checkpoint kinases from *S. pombe* and *Drosophila.*

Chk1 of *S. pombe* has significant homology to the grapes protein kinase of *Drosophila* (6). Both proteins are involved in delaying cell cycle progression in the presence of unreplicated or damaged DNA (6, 7, 8). We therefore searched for a human homologue of the chk1/grapes kinase. A human expressed sequence tag encoding a protein with homology to chk1/grapes was identified by BLAST analysis of the NCBI maintained database. The human cDNA, designated hChk1 for human checkpoint kinase 1, contained a predicted open-reading frame of 476 codons and encoded a protein of approximately 56kDa. The same protein was identified by Sanchez et al. (9).

Human Chk1 is functionally related to checkpoint kinases from *S. pombe* and *Drosophila.*

To determine if hChk1 is functionally equivalent to chk1/grapes a HeLa cell lysate assay that measures the effect of exogenously added proteins on Cdc2 phosphorylation was used. In particular, the ability of recombinant proteins produced by baculovirus infection of Sf9 cells, to alter Cdc2 phosphorylation was tested. Lysates from mitotic HeLa cells were mixed with extracts from Sf9 cells containing Cdc25C, Wee1, Myt1 or hChk1 in the presence of an ATP-regenerating system. The results of such analysis are shown in Figure 2A. Three molecular weight species corresponding to the unphosphorylated, singly phosphorylated and the doubly phosphorylated Cdc2 can readily be detected in the samples that were treated with recombinant Myt1 and/or Wee1. Increased phosphorylation of Cdc2 correlated with the loss of activity seen in histone H1 kinase assays. Wee1 and Myt1 are both known to phosphorylate and inactivate Cdc2 (10, 11). The control sample, which was treated with Sf9 extracts from uninfected cells, contained a small proportion of phosphorylated Cdc2, this was most probably derived from the ~10% interphase cells that were present in this preparation of mitotic HeLa cells. The presence

of phosphorylated Cdc2 in this extract allowed dephosphorylation and activation of Cdc2 to be monitored on addition of exogenous Cdc25. Thus both the kinases and phosphatases that have opposing control over Cdc2 can be active in this assay system. The loss of histone H1 kinase activity, relative to the control sample, seen on addition of hChk1, correlated with the maintained presence of a small amount of phosphorylated Cdc2. This experiment shows that hChk1 is able to impact the phosphorylation state of cdc2 and it is therefore functionally, as well as structurally, related to *S. pombe* chk1 and *Drosophila* grapes. It is not possible from this experiment to conclude whether hChk1 is promoting phosphorylation of Cdc2 by activating Wee1/Myt1 or by inhibiting Cdc25.

Human Chk1 inhibits dephosphorylation of Cdc2 *in vitro*

A two part assay in which hChk1 was first able to phosphorylate substrate proteins in the presence of ATP, followed by an assay for phosphatase activity in the presence of EDTA was used to determine if Cdc25 activity was affected by the hChk1 kinase. Extracts from thymidine arrested HeLa cells (which contain inactive Cdc2/Cyclin B complex) were first incubated with or without hChk1 in the presence of an ATP regenerating system. EDTA was added and 30 minutes later the activation state of Cdc2 was monitored. As shown in Figure 2B, an 20-fold increase in Cdc2 activity was seen in control samples following addition of EDTA, activation correlated with the loss of the phosphorylated Cdc2 seen by western analysis. By contrast, extracts that contained hChk1 showed only a 2-fold increase in Cdc2 activity. The reduced phosphatase activity was dependent on hChk1 activity; it was not seen when a kinase inactive mutant (hChk1 K/R 38) was used and it was not seen when ATP was not present in the reaction (data not shown). Confirmation that Cdc2 was not irreversible inhibited by hChk1 was shown by the dephosphorylation and activation that result when excess exogenous Cdc25 is added after hChk1 treatment.

Three Cdc25 species are known to exist in mammalian cells (12), Cdc25C is thought to be the dominant phosphatase acting on Cdc2/Cyclin B(13, 14), however a role for Cdc25A and B in the regulation of Cdc2 has not been excluded. Inhibition of the dephosphorylation of Cdc2 could be due to direct inhibition of Cdc25 activity, by degradation, by prevention of the Cdc2 dependent activation of Cdc25 that normally occurs in M-phase (14, 15) or interference in the accessibility of Cdc2 to Cdc25. It is difficult to imagine that an effect on sub-cellular localisation could be measured in cell lysates that have been clarified by centrifugation at 10,000 x g. The abundance of Cdc25C is not effected by DNA damage *in vivo* (14, 15) and loss of Cdc25C from hChk1 treated extracts was not seen in this study (data not shown). Therefore degradation of Cdc25 is not likely to account for the reduced dephosphorylation of Cdc2. Activation of Cdc2 was not seen in the presence of an ATP-regenerating system (data not shown, but note that the starting activity in Figure 1 and 3 are equivalent), therefore in this assay scheme, Cdc2 was not able to phosphorylate Cdc25. Thus, the Cdc2 dependent activation of Cdc25 that normally occurs in M-phase (14, 15) could not have contributed to the difference in Cdc25 activity seen in control and hChk1 treated samples. Thus, the decreased rate of dephosphorylation can only be easily explained by a reduced activity of the major phosphatase (Cdc25C) that dephosphorylates Cdc2.

The reduced activation of Cdc2 seen following addition of hChk1 suggested that hChk1 might be acting in a similar manner to irradiation in inhibiting the rate of dephosphorylation of Cdc2. We therefore repeated the time course experiment shown in Figure 1. hChk1 was added to HeLa cell lysates in the presence of an ATP regenerating system for 30 minutes, EDTA was added, and the rate of Cdc2 dephosphorylation was monitored. Data from this experiment, shown in Figure 3, suggest that the major phosphatase acting on Cdc2/Cyclin B is inhibited by hChk1. This assay does not address the possibility that Wee1/Myt1 are also substrates that might be activated by hChk1 or that other Cdc2 directed phosphatases are also be inhibited.

Cdc25 and Wee1 are substrates for hChk1 *in vitro*

To determine if Wee1, Myt1 or Cdc25 could be direct targets of hChk1 we purified the proteins from baculo-infected cells and tested the ability of each protein to act as a substrate for hChk1 *in vitro*. As shown in Figure 4 both Wee1 and Cdc25 are substrates for hChk1 *in vitro*. Myt1 was not detectable phosphorylated in the same assay system (data not shown). Despite the fact that hChk1 directly phosphorylates Cdc25 *in vitro* we were unable to demonstrate that hChk1 directly inactivates Cdc25. This observation is not wholly explained by the recent report that the site on Cdc25 phosphorylated by hChk1, S216, is a 14-3-3 binding motif (16). Genetic data indicate a role for 14-3-3 proteins in the DNA damage checkpoint of *S. pombe* (17). However, even when 14-3-3 proteins are provided inactivation of Cdc25 by hChk1 has not be reproduced *in vitro* using purified components ((16) and data not shown). This suggests that the HeLa cells lysate used in this study contribute a third, unknown factor that is required for the inactivation of Cdc25. Experiments are underway to further investigate this possibility and to determine whether phosphorylation of Wee1 by hChk1 has any effect on activity.

B) GENERATION OF SUBSTRATES FOR WEE1 AND CDC25 ASSAYS.

As outlined last year we have had a number of technical difficulties in improving the assays for Wee1 and Cdc25. The strategy which we are currently using is the produce tagged-CDC2 in baculo-infected SF9 cells. The Bac-to Bac system (Gibco) was used to isolate a set of viruses that direct expression of wild-type CDC2 and the phosphorylation site mutants. These proteins have been produced in single infections or co-produced in the presence of un-tagged CyclinB, and complex isolated on NTA-Ni agarose beads. We have found that by using kinase inactive Cdc2 (K/R33) we can reduce the background phosphorylations such that the activity of Wee1 can be readily measured in immune-precipitates from asynchronous cells, and as expected, not in immune-precipitates from mitotic cells (Data not shown). This assay will now be used to determine if Wee1/Myt1 activity is increased in response to DNA damage and to determine whether hChk1 is involved in any change in activity.

7) Conclusion

The results obtained from our analysis of the over-expression of non-phosphorylatable CDC2 in HeLa cells validate the hypothesis on which much of the proposal was based. We therefore continued with experiments aimed at showing whether Wee1/Myt1 or Cdc25 activity is altered in response to DNA damage. We found that Cdc25 activity is reduced following irradiation and have identified a human kinase hChk1 which can phosphorylate and inactivate Cdc25. Wee1 was also found to be a substrate for hChk1 however we do not know whether its activity is altered by phosphorylation.

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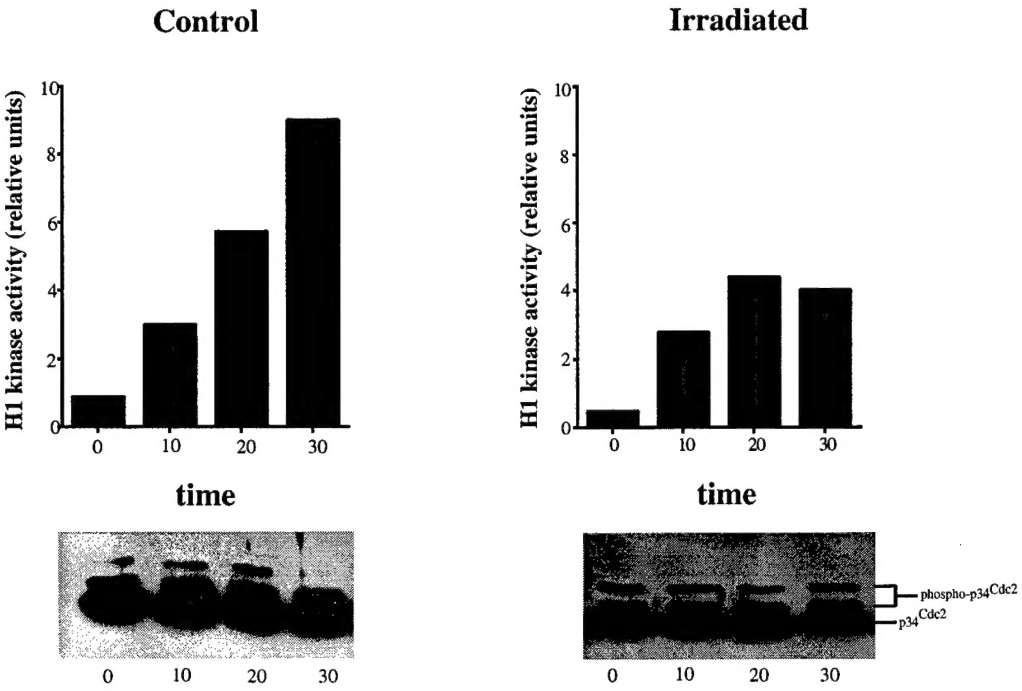
Figure 1



Dephosphorylation of cdc2 is reduced following irradiation of HeLa cells.

A culture of asynchronous HeLa cells was divided, and either not treated or irradiated with 10 Grey 1 hour prior to harvesting. Cells were lysed in ice-cold lysis buffer (50mM Tris pH 7.4 containing 10 mM magnesium chloride, 1 mM phenylmethylsulphonyl fluoride, and 5 µg/ml leupeptin, pepstatin and aprotinin). Lysates were cleared by centrifugation at 10,000 x g for 10 minutes and the protein concentration of the supernatants determined using the Lowry assay. 10mM EDTA was added to the supernatants (100µg in 60µL) and the reaction initiated by incubation at 30oC. At the indicated time the activity of Cdc2/Cyclin B was assayed by measuring the histone H1 kinase activity present in anti-cyclin B immune-precipitates. The phosphorylation state of Cdc2 was assayed by immune-blotting using an anti-Cdc2 specific antibodies.

Figure 2A

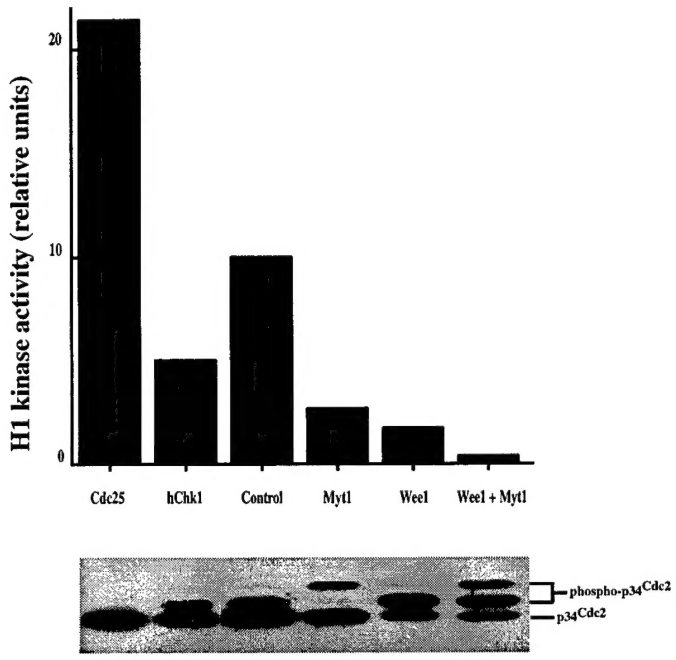
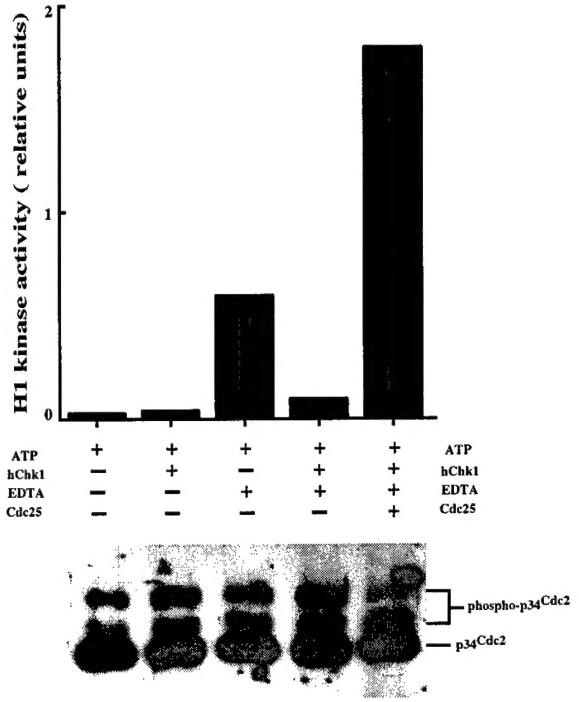


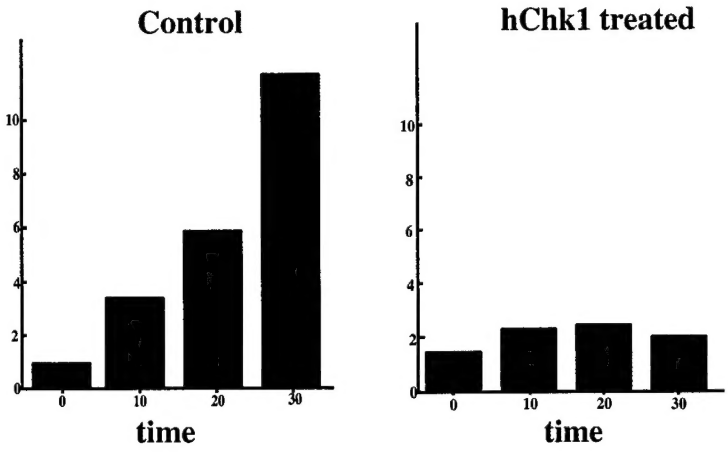
Figure 2B



hChk1 affects the phosphorylation state of Cdc2 *in vitro*

Panel A, cell lysates from mitotic HeLa cells were incubated with lysates of Sf9 cells infected with viruses encoding 6his-Cdc25, 6his-Wee1, 6his-Myt1 or 6his-hChk1 in the presence of an ATP-regenerating system. After 30 minutes at 30oC the reaction was terminated and the activity and phosphorylation state of Cdc2 was assayed as in Figure 1. Panel B, lysates from thymidine arrested HeLa cells incubated with Sf9 cell lysates infected with viruses encoding 6his-hChk1 or uninfected cells, in the presence of an ATP regenerating system. After 30 minutes at 30oC , EDTA and/or Cdc25 was added and samples kept at 30oC for a further 30 minutes. The activity and phosphorylation state of Cdc2 was then assayed

Figure 3



hChk1 inhibits dephosphorylation of Cdc2
Lysates prepared from asynchronous cells were incubated with Sf9 cell lysates expressing hChk1 or with lysates from uninfected cells in the presence of an ATP-regenerating system at 30oC. After 30 minutes EDTA was added and the activation of Cdc2 was monitored.

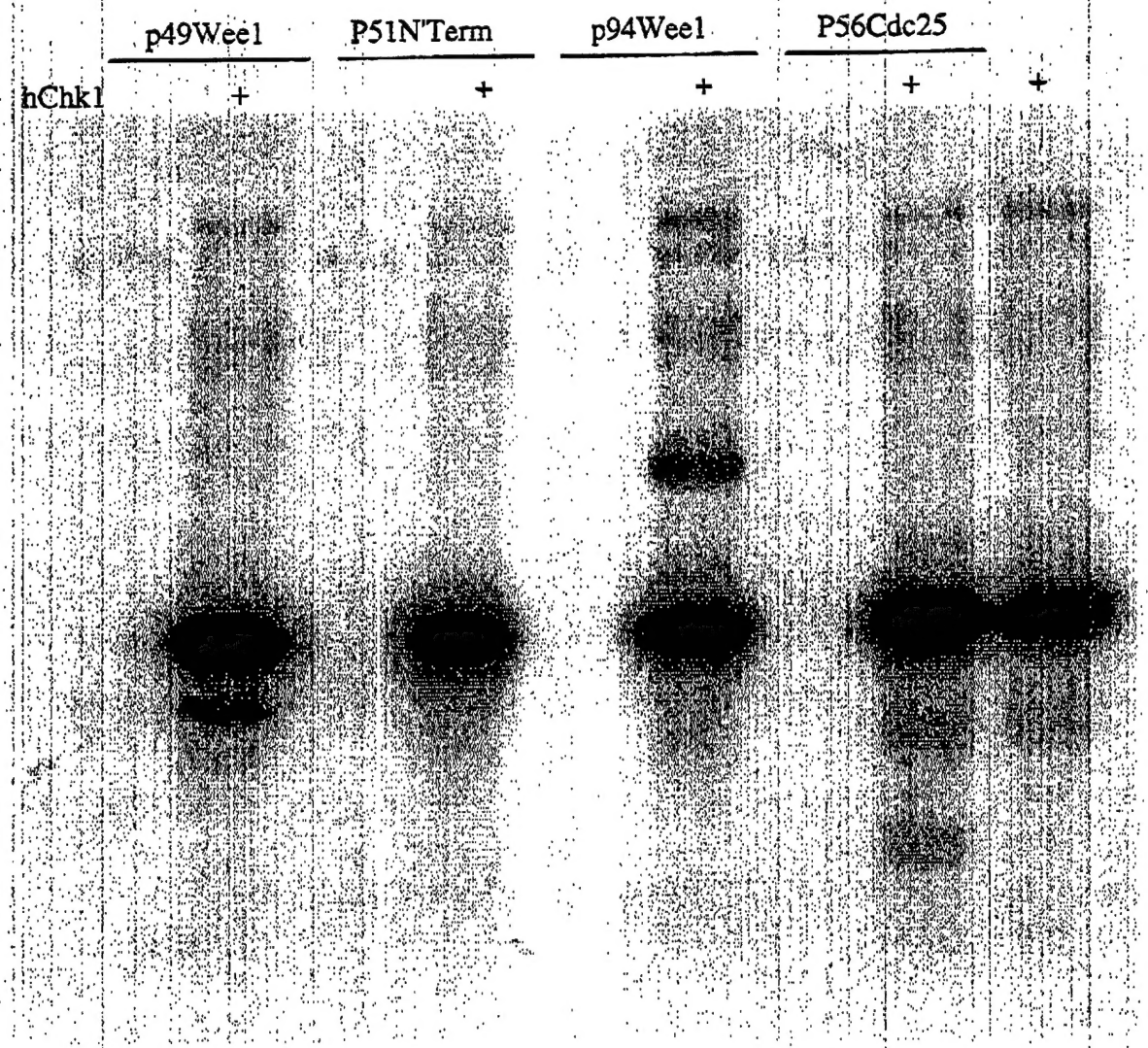


Figure 4
hChk1 Phosphorylates the C' terminus of Wee1 in vitro.

hChk1, p49 C' terminal Wee1, p51N' terminal Wee1, p94Wee1, p56Cdc25c and p56Chk1 were produced as 6his fusion proteins in baculo-infected Sf9 cells. The proteins were purified by NiNTA chromatography, incubated in the presence of 32P-γ-ATP, the products of the in vitro kinase reaction were analyzed by SDS-PAGE.